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Immunization with High-Molecular-Weight Adhesion Proteins of Nontypeable *Haemophilus influenzae* Modifies Experimental Otitis Media in Chinchillas

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Prevention of nontypeable *Haemophilus influenzae* otitis media by vaccination is an important health care goal. Proteins important in bacterial adherence deserve consideration as potential vaccine candidates. Two colleagues and I previously identified a family of immunogenic high-molecular-weight proteins important in adherence of nontypeable *H. influenzae* to human epithelial cells (J. W. St. Geme III, S. Falkow, and S. J. Barenkamp, Proc. Natl. Acad. Sci. USA, 90:2875–2879, 1993). In the work described here, I determined whether immunization with two such adherence proteins, HMW1 and HMW2, purified from prototype nontypeable *Haemophilus* strain 12, would modify the course of experimental otitis media caused by the homologous strain. Chinchillas received three monthly subcutaneous injections with 40 μg of an HMW1/HMW2 protein mixture in Freund's adjuvant. One month after the last injection, animals were challenged by intrabullar inoculation with 300 CFU of nontypeable *H. influenzae* 12. Infection developed in five of five control animals versus 5 of 10 immunized animals ($P = 0.08$, Fisher exact, one-tailed). Among infected animals, bacterial counts in middle ear fluid specimens 7 days postchallenge were significantly greater in control animals than in immunized animals ($P = 0.014$, Mann-Whitney U test). Serum antibody titers following immunization were comparable in uninfected and infected animals. However, infection in immunized animals was uniformly associated with the appearance of bacteria downregulated in expression of the high-molecular-weight proteins, suggesting bacterial selection in response to immunologic pressure. Although protection following immunization was incomplete, these data suggest that the high-molecular-weight adhesion proteins are potentially important protective antigens which might represent one component of a multicomponent nontypeable *Haemophilus* vaccine.

Otitis media remains an important health problem for children in this country and elsewhere in the world. Most children have had at least one episode of otitis by the third birthday, and one-third have had three or more episodes (41). Bacteria, usually in pure culture, can be isolated from middle ear exudates in approximately two-thirds of cases of acute otitis media. *Streptococcus pneumoniae* is the most common bacterial agent in all age groups, having been cultured from approximately 40% of acute effusions (10, 15, 23). Nontypeable *Haemophilus influenzae* generally accounts for 20 to 25% of cases of acute otitis media and for a larger percentage of cases of chronic otitis media with effusion (10, 15, 23).

Host immunity plays an important role in the prevention of disease (14, 35, 38). During middle ear infection, immunoglobulins, complement components, and phagocytic cells are all found within the middle ear space. In addition, serum and middle ear fluid antibodies directed against the infecting organisms develop during the course of bacterial otitis media (8, 9, 13, 14, 37). In cases of nontypeable *Haemophilus* otitis media, the presence of these antibodies is associated both with decreased numbers of bacteria in the middle ear fluid (13) and with more rapid resolution of infection (9, 35). These data suggest that it may be possible to impact the incidence or severity of nontypeable *Haemophilus* otitis media by vaccination of susceptible individuals (33). However, which bacterial components should be included in a nontypeable *H. influenzae* vaccine is unclear at present.

In previously reported work, a family of high-molecular-weight proteins which are major targets of antibody in sera of children recovered from *Haemophilus* otitis was identified (4). Furthermore, a strong correlation between the appearance of or increase in serum antibodies directed against these high-molecular-weight proteins and the development of bactericidal antibody was observed (4). Subsequently, the genes encoding two such immunogenic high-molecular-weight proteins from a prototypic strain were cloned and sequenced (5), and it was demonstrated that the proteins encoded by these genes were critical for attachment of nontypeable *H. influenzae* to human epithelial cells in vitro (39). Given the functional role of these proteins as adhesins and their highly immunogenic character, it was reasoned that the high-molecular-weight proteins warranted consideration as possible vaccine candidates. The present report describes studies examining the protective potential of the *Haemophilus* high-molecular-weight adhesion proteins in the chinchilla model of otitis media.

MATERIALS AND METHODS

Bacterial strain. Nontypeable *H. influenzae* strain 12 was the clinical isolate chosen as the prototypic strain for study (5). This organism was isolated in pure culture from the middle ear fluid of a child with acute otitis media. The strain was identified as *H. influenzae* by standard methods (25) and classified as nontypeable by its failure to agglutinate with a panel of typing antisera for *H. influenzae* types a to f (Burroughs Wellcome Co., Research Triangle Park, N.C.) and failure to show lines of precipitation with these antisera in counterimmunoelectrophoresis assays (42). Furthermore, genomic DNA purified from strain 12 failed to hybridize with a plasmid probe, pUO38, which contains DNA from the *cap* region of type b *H. influenzae* (12). The organism was maintained at −70°C in skim milk after two subpassages from the initial clinical isolation.

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Growth conditions of bacteria for animal challenge. Bacteria were recovered from skim milk stocks by transfer of a loopful of thawed organisms to a chocolate agar plate and incubation for 16 h at 37°C in a 5% CO_2 atmosphere. The next day, 5 to 10 colonies were isolated with a sterile loop and used to inoculate 50 ml of brain heart infusion (BHI) broth supplemented with NAD and hemin, each at 10 μg/ml. Growth proceeded for 4 to 6 h at 37°C in 250-ml Erlenmeyer flasks with a shaker-incubator (model G25; New Brunswick Scientific Co., Inc., Edison, N.J.). Bacteria in mid-log phase with an A_{600} of 0.5 to 0.6 were harvested by centrifugation at 12,000 × *g* at 4°C and washed twice with phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and $CaCl_2$ and $MgCl_2$ at final concentrations of 0.15 and 0.5 mM, respectively. The washed bacterial cells were maintained at 0°C for less than 1 h before being used for animal challenge as described below.

Purification of native high-molecular-weight adhesion proteins. The native high-molecular-weight adhesion proteins HMW1 and HMW2 were purified from prototypic nontypeable *Haemophilus* strain 12 as follows. The frozen bacterial stock culture was streaked onto a chocolate plate and allowed to grow overnight at 37°C as described above. The following day, a 50-ml starter culture of BHI broth supplemented with hemin and NAD was inoculated with 5 to 10 colonies. The starter culture was shaken at 37°C in a rotary incubator at 250 rpm until the optical density of the culture reached an A_{600} of 0.6 to 0.8. Six 500-ml flasks of supplemented BHI broth were then inoculated with 8 to 10 ml of the bacterial suspension from the starter culture and allowed to grow to an optical density of 1.2 to 1.5. Bacterial cells from the six flasks were pelleted by centrifugation at 12,000 × *g* for 10 min at 4°C and frozen overnight at −20°C in preparation for purification of the proteins.

The following day, the bacterial pellets from the six flasks were resuspended uniformly and combined in 250 ml of extraction solution, consisting of 0.5 M NaCl, 0.01 M disodium EDTA, 0.01 M Tris, and 50 μM 1,10-phenanthroline, pH 7.5. The bacterial cells were not sonicated or otherwise mechanically disrupted, but simply resuspended and allowed to incubate at 0°C for 60 min. The bacterial suspensions were then centrifuged at 12,000 × *g* for 10 min at 4°C to remove the majority of intact cells and cellular debris. The supernatant, containing the water-soluble high-molecular-weight adhesion proteins, was then subjected to centrifugation at 100,000 × *g* for 60 min at 4°C to remove membrane fragments and additional debris. The supernatant from this ultracentrifugation step, containing the high-molecular-weight proteins, was dialyzed overnight at 4°C against 0.01 M sodium phosphate, pH 6.0.

The following day, the sample was centrifuged at 12,000 × *g* for 10 min at 4°C to remove insoluble debris, which sometimes precipitated from the solution during overnight dialysis. The supernatant was then applied to a 10-ml CM Sepharose column (Sigma Chemical Co., St. Louis, Mo.) which had been equilibrated with 0.01 M sodium phosphate, pH 6.0. Following application of the protein-containing sample, the column was washed with 2 column volumes of 0.01 M sodium phosphate, and the proteins were eluted with a 0 to 0.5 M KCl gradient. Column fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to identify fractions containing high-molecular-weight proteins. Column fractions containing the high-molecular-weight proteins were pooled, concentrated to a volume of 1 to 3 ml, and maintained at 0°C overnight.

The following day, the sample was applied to a Sepharose CL-6B (Sigma) gel filtration column equilibrated with PBS, pH 7.5. Column fractions containing high-molecular-weight proteins free of contamination by lower-molecular-weight species were identified by SDS-PAGE. The relevant fractions were pooled and stored at −70°C in preparation for the immunization experiments.

Experimental animals. Healthy adult chinchillas 1 to 2 years of age and weighing 350 to 500 g were used for immunization and challenge experiments. The animals were obtained from an outbred chinchilla colony maintained at Washington University School of Medicine by the Department of Otolaryngology. The animals were housed in separate cages throughout the experiments.

Animal immunization and experimental infection. The group of experimental animals received three monthly subcutaneous injections with 40 μg of the HMW1/HMW2 protein mixture in Freund's adjuvant. Control animals received PBS in Freund's adjuvant. The first doses of either purified HMW1/HMW2 or PBS were mixed with Freund's complete adjuvant, and subsequent doses were mixed with incomplete Freund's adjuvant. Serum antibody responses were monitored by enzyme-linked immunosorbent assay (ELISA) as described below with the purified HMW1/HMW2 mixture as the antigen and rabbit anti-chinchilla immunoglobulin G (IgG)/IgM as the antiserum used to detect bound chinchilla antibody (3). Serum antibody responses were also monitored by measurement of agglutinating activity as described below. Sera were collected from each animal prior to the start of the immunization series and immediately prior to bacterial challenge.

One month after the last immunization, animals were challenged by direct middle ear inoculation with 300 CFU of nontypeable *Haemophilus* strain 12. For bacterial challenge, freshly grown strain 12 cells were prepared as described above and diluted to a concentration of 10^4 CFU/ml in PBS–0.5% bovine serum albumin (BSA) with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$, pH 7.4. The colony count was confirmed by counting appropriate dilutions. The left middle ear space of each experimental animal was then inoculated via the epitympanic bulla with 0.1 ml of the bacterial suspension (containing 10^3 CFU). This bacterial inoculum causes middle ear infection in 100% of nonimmune animals.

The course of middle ear disease was monitored by otoscopy, aspiration of fluid from the middle ear space via the epitympanic bullae, and quantitative culture of collected fluid. Middle ear aspiration was performed every 2 to 3 days after bacterial challenge irrespective of whether otoscopic examination demonstrated the presence of tympanic membrane inflammation or middle ear effusion.

ELISA measurement of antibodies directed against purified high-molecular-weight adhesion proteins. Chinchilla serum antibody directed against the native HMW1 and HMW2 proteins was determined by ELISA. In brief, 96-well flat-bottomed enzyme immunoassay microtitration plates (Linbro/Titertek; Flow Laboratories, Inc., McLean, Va.) were coated overnight at 4°C with a purified HMW1/HMW2 mixture (10 μg of total protein per ml) in $NaCO_3$ buffer, pH 9.6. The following day, the plates were blocked with PBS–0.5% BSA at room temperature for 1 h. The plates were then washed with PBS–0.5% BSA–0.05% Tween 20 prior to addition of the chinchilla serum specimens. The chinchilla sera were diluted in PBS–0.5% BSA–0.05% Tween 20 and incubated for 1 h at room temperature. After additional washes, the wells were incubated with a 1:500 dilution of rabbit anti-chinchilla IgG/IgM antiserum. This serum was prepared as described previously (3). This incubation was also carried out for 1 h at room temperature. Following additional washes, the wells were incubated with a 1:3,000 dilution of IgG-specific goat antibody to rabbit immunoglobulin conjugated to alkaline phosphatase (Bio-Rad, Hercules, Calif.), with this latter incubation also carried out for 1 h at room temperature. After additional washes, the wells were incubated with 200 μl of a 1-mg/ml solution of *p*-nitrophenyl phosphate in 10% diethanolamine buffer (pH 9.8). Absorbance was monitored at 405 nm with a Titertek multiscan spectrophotometer (Flow Laboratories).

Preimmune and immune sera from all immunized animals were assayed simultaneously. Serial dilutions of a chinchilla immune serum pool raised against strain 12 high-molecular-weight proteins were run as a control with each HMW1/HMW2 ELISA experiment. The test reactions were read when a 1:25,000 dilution of this positive control reached an optical density of 0.3. Negative controls included wells coated with antigen and reacted with alkaline phosphatase conjugate alone; mock-coated wells incubated with $NaCO_3$ buffer without added protein, which were then reacted with serum and conjugate; and mock-coated wells reacted with conjugate alone. All negative controls gave optical density readings of <0.1.

Measurement of serum agglutinating activity. The ability of sera to mediate agglutination of bacteria was determined as follows. Freshly grown bacteria recovered from overnight growth on chocolate plates were resuspended in PBS–0.5% BSA to an optical density of 0.5. A mixture of 50 μl of the bacterial suspension and 10 μl of the serum or serum sample dilution was then mixed in wells on an agglutination plate and shaken on a rotary shaker for 5 min. Agglutination was monitored by examining the sample with a light microscope. The agglutination titer was defined as the highest dilution of serum mediating visible clumping of the bacteria under the microscope. Negative controls included bacteria incubated with PBS–0.5% BSA alone and bacteria incubated with nonimmune chinchilla sera.

Western immunoblot assay. Chinchilla sera were analyzed by Western immunoblot analysis to monitor the antibody responses to specific *Haemophilus* antigens. Nontypeable *H. influenzae* strain 12 cell sonicates containing 100 μg of total protein were solubilized in electrophoresis sample buffer, subjected to SDS-PAGE on 7.5% acrylamide gels, and transferred to nitrocellulose with a Genie electrophoretic blotter (Idea Scientific Company, Corvallis, Oreg.) for 45 min at 24 V. After transfer, the nitrocellulose sheet was blocked and then probed sequentially with the chinchilla sera, with rabbit antiserum to purified chinchilla IgG/IgM, and with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Bio-Rad), and finally bound antibodies were detected by incubation with nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) solution.

A slight variation of this assay was employed to examine the expression of the HMW1 and HMW2 proteins of nontypeable *Haemophilus* strains isolated from middle ear fluid specimens of infected chinchillas. In this assay, cell sonicates were prepared and transferred to nitrocellulose as described above. The membranes were probed with rabbit antiserum raised against recombinant HMW protein prepared as described previously (5) and then probed with alkaline phosphatase-conjugated goat anti-rabbit IgG prior to incubation with the developing solution.

Statistical analysis. The working hypothesis in the design of the study was that parenteral immunization with the high-molecular-weight proteins would modify the course of disease in immunized animals, as evidenced either by complete protection of the immunized animals or by decreased bacterial density in the middle ear effusions which did develop. Sample size was calculated by assuming a 100% rate of infection of control animals and at least 50% complete protection of immunized animals. The Fisher exact test was employed to compare the proportion of animals infected in the control and immunized groups. To compare the middle ear fluid bacterial densities in infected animals in the control and immunized groups, the data were log transformed and then compared with the Mann-Whitney U test.

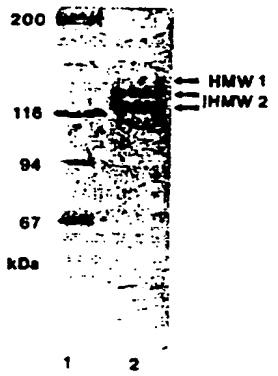

FIG. 1. Coomassie blue-stained 7.5% SDS-PAGE gel with purified high-molecular-weight proteins from prototypic nontypeable *H. influenzae* strain 12. The arrows indicate the identity of each visualized protein band, based upon comparison of the N-terminal amino acid sequence of the indicated band with the derived amino acid sequences of the cloned *hmw1A* and *hmw2A* structural genes (5). Lane 1, molecular size standards (in kilodaltons).

RESULTS

Purification of native high-molecular-weight adhesion proteins. Development of the purification protocol for the nontypeable *Haemophilus* strain 12 HMW1 and HMW2 adhesion proteins was guided by several facts and experimental observations. As previously reported, the HMW1 and HMW2 proteins demonstrate a number of structural and functional similarities with the filamentous hemagglutinin protein of *Bordetella pertussis* (5, 39). A number of purification protocols have been developed for the latter protein (1, 34). I reasoned that these protocols might be applicable to purification of the *Haemophilus* proteins as well. In addition, immunoelectron microscopy studies of the *Haemophilus* proteins had demonstrated them to be abundantly expressed on the bacterial surface but only loosely attached (2). Thus, I reasoned that purification protocols that released surface-associated proteins without disrupting the bacterial cells as a whole should result in a preparation enriched in the proteins of interest. Application of these ideas ultimately led to the purification protocol outlined in detail in Materials and Methods.

Figure 1 shows a an SDS-PAGE gel with the HMW1 and HMW2 protein mixture recovered in water-soluble form from nontypeable *Haemophilus* strain 12. The HMW1 and HMW2 proteins copurify by the method described. Approximately 1 mg of the purified protein mixture was recoverable per liter of late-log-phase bacterial cells. HMW1 has an apparent molecular mass of 125 kDa. HMW2 has an apparent molecular mass of 120 kDa. The third band seen, with an apparent molecular mass of 118 kDa, is a partial degradation product of the HMW2 protein. The identity of each band was confirmed by N-terminal amino acid sequence analysis and comparison of that data with the predicted amino acid sequences of the cl ned structural genes (5). The 125-kDa band had an N-terminal amino acid sequence of --NV-INEATAG, corresponding to the derived amino acid sequence of the *hmw1A* structural gene, beginning at residue 442. The 120- and 118-kDa bands both demonstrated N-terminal amino acid sequences of P--VTIE-AEDPL, corresponding to the derived amino acid sequence of the *hmw2A* structural gene, also beginning at residue 442. These data suggest that lengthy segments are removed from the amino termini of the structural gene products in formation of the mature proteins. As can be appreciated from this figure, the amount of HMW1 protein recovered in the final preparation is much less than that of the two HMW2 species. The reasons for this difference in abundance of the two protein species are unclear.

TABLE 1. ELISA and agglutination titers of PBS- and HMW1/HMW2-immunized chinchillas

Antigen	No. of animals	Median titer (range)	
		ELISA	Agglutination assay
PBS	5	<100	<10
HMW1/HMW2	10	100,000 (50,000–200,000)	500 (250–1,000)

Immunization of experimental animals and determination of antibody responses. Ten experimental animals were immunized with the purified HMW1/HMW2 protein mixture with adjuvant. Animals received 40-μg doses administered subcutaneously every 4 weeks for 2 months. The five control animals were immunized with PBS with adjuvant on the same schedule. One month following the third immunization, the antibody responses of each animal were determined in ELISA, bacterial agglutination, and Western immunoblot assays.

Table 1 summarizes the results of the ELISA and bacterial agglutination assays performed with sera from the immunized animals. All animals lacked ELISA-detectable antibody to the purified high-molecular-weight proteins in the preimmunization sera. In addition, no serum-agglutinating activity was detectable in any of the preimmune sera. The PBS-immunized animals also lacked ELISA-detectable antibody and serum-agglutinating activity in their postimmunization sera. In contrast, all animals immunized with the purified high-molecular-weight proteins demonstrated high-titer ELISA antibody against the purified proteins, with titers ranging from 50,000 to 200,000. In addition, these animals developed very high levels of agglutinating antibody, with titers ranging from 250 to 1,000, and a median agglutinating titer of 500.

When examined by Western immunoblot assay against a cell sonicate of nontypeable *Haemophilus* strain 12, immune sera of HMW1/HMW2-immunized animals demonstrated antibodies directed almost exclusively against the HMW1 or HMW2 protein (Fig. 2, large arrow). Occasional animals demonstrated low-level activity against a lower-molecular-weight species (Fig. 2, small arrow), corresponding in size to the minor contaminating protein band noted in the HMW1/HMW2 immunizing preparation.

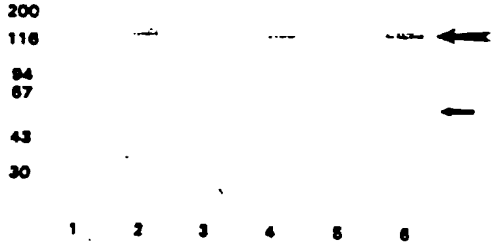

FIG. 2. Western immunoblot assay with cell sonicates of nontypeable *H. influenzae* strain 12 probed with preimmune and immune sera of chinchillas immunized with the HMW1/HMW2 purified protein preparation. Shown are paired preimmune (lanes 1, 3, and 5) and immune (lanes 2, 4, and 6) sera from three representative animals. The large arrow indicates the location of the two bands corresponding to HMW1 and HMW2. The small arrow indicates the location of a band corresponding to the lower-molecular-weight protein species which was a minor contaminant of the HMW1/HMW2 preparation. Sizes are shown in kilodaltons.

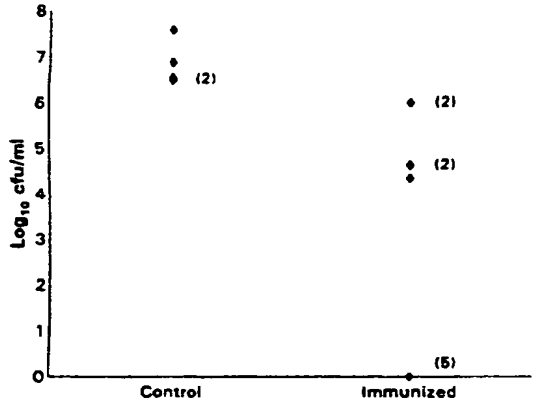

FIG. 3. Middle ear fluid bacterial counts in PBS-immunized control animals (n = 5, left panel) and HMW1/HMW2-immunized animals (n = 10, right panel) 7 days after middle ear inoculation with nontypeable *H. influenzae* strain 12. Numbers in parentheses indicate the number of animals at each point (>1).

Challenge experiments with prototypic nontypeable strain 12. Once antibody responses were demonstrated, all experimental animals were subjected to middle ear bacterial challenge. Five of five control animals developed culture-positive otitis media, with positive middle ear fluid cultures and tympanic membrane inflammation persisting for approximately 2 weeks. In contrast, only 5 of the 10 high-molecular-weight protein-immunized animals developed culture-positive otitis (P = 0.08, Fisher exact, one-tailed). The five HMW1/HMW2-immunized animals that did not develop otitis never demonstrated signs of middle ear inflammation when examined by otoscopy, nor were middle ear effusions detectable.

Among the five HMW1/HMW2-immunized animals that became infected, the total duration of middle ear infection, as assessed by the persistence of culture-positive middle ear fluid, was not different from that in the controls. However, the degree of inflammation of the tympanic membranes was subjectively less in the HMW1/HMW2-immunized animals. When quantitative bacterial counts were performed on the middle ear fluid specimens recovered from infected animals, notable differences were apparent between the HMW1/HMW2-immunized and PBS-immunized animals (Fig. 3). Shown in this figure are quantitative middle ear fluid bacterial counts from animals on day 7 postchallenge, a time point associated with maximum colony counts in middle ear fluid. The data from the c ntrol animals are shown on the left, and data from the high-molecular-weight protein-immunized animals are on the right. The HMW1/HMW2-immunized animals had significantly lower middle ear fluid bacterial counts than the PBS-immunized controls (P = 0.005, Mann-Whitney U test). Furthermore, the difference remained significant even when the immunized animals fully protected against infection were excluded from the analysis (P = 0.014, Mann-Whitney U test).

The mean serum antibody titers of the HMW1/HMW2-immunized animals that developed otitis were not different from those of the immunized but protected animals. Thus, differences in antibody levels could not explain why some animals developed infection and others did not. An alternative explanation was that the bacteria causing infection may have changed phenotypically. T explore this possibility, I examined bacteria recovered from middle ear fluid specimens of c ntrol and HMW1/HMW2-immunized animals for expression of the two high-molecular-weight proteins. Figure 4 shows a Western blot in which typical isolates recovered from the two groups of animals are compared. As can be seen, the 125-kDa HMW1 band of strains recovered from both control and HMW1/HMW2-immunized animals appeared to be relatively normal in intensity. In contrast, the 120-kDa HMW2 band was markedly decreased in intensity or absent in all strains recovered from HMW1/HMW2-immunized animals. As was noted earlier, HMW2 was the major component of the purified protein preparation used for immunization (Fig. 1). In the PBS-immunized control animals, the bacteria recovered from middle ear fluid specimens demonstrated normal expression of both high-molecular-weight proteins (Fig. 4).

When the strains recovered from infected animals were examined in the agglutination assay, the bacteria downregulated in HMW2 expression could no longer be agglutinated by the immune sera of HMW1/HMW2-immunized chinchillas. This suggested that HMW2 was the major target of agglutinating antibody elicited by immunization. The HMW1 protein pr s-ent in the immunizing preparation was apparently in ffective in generating significant levels of agglutinating antibody, given the small amount present compared with the amount of HMW2 (Fig. 1). The bacteria recovered from middle ear fluid specimens of the control animals could be agglutinated by the chinchilla immune sera at titers equivalent to those seen above with the parent strain used for middle ear challenge. Thus, these data suggest that in this experimental model, nontypeable *Haemophilus* variant strains downregulated in high-molecular-weight protein expression rapidly appear in the face of immune pressure.

Immunization experiment with purified HMW1. In arlier work, it was reported that HMW1 appeared to be the more important adhesin when strain 12 binding was examined in vitro with Chang conjunctival epithelial cells (39). It was reasoned that if sufficient antibody to HMW1 could be raised in the immunized animals, then more complete protection might be demonstrated. Therefore, HMW1 was purified from a mutant strain deficient in expression of HMW2 (39), and an additional active immunization experiment in which HMW1 alone was used as the immunizing antigen was performed. The results of this latter experiment mirrored those described above.

It was possible to fully protect 3 of 12 HMW1-immunized animals against infection with nontypeable *Haemophilus* strain 12. In contrast to the experiment described above, quantitative differences in middle ear fluid bacterial densities were not apparent when immunized and control animals were com-

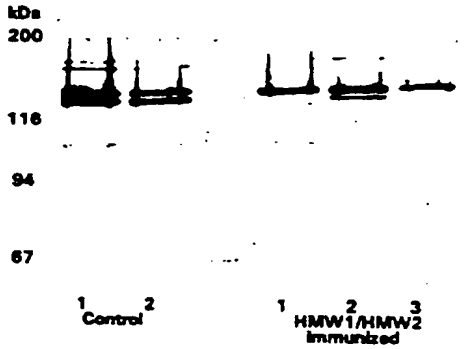

FIG. 4. Western immunoblot assay with cell sonicates of nontypeable *H. influenzae* strain 12 bacteria recovered from middle ear fluid specimens of two control animals immunized with PBS or three animals immunized with the HMW1/HMW2 protein mixture. The sonicates were probed with rabbit antiserum prepared against the HMW1/HMW2 proteins as described previously (5).

pared. However, similar to the findings noted above, when the bacteria recovered from middle ear fluid specimens were examined for high-molecular-weight protein expression by Western blot analysis, phenotypic changes were noted. Specifically, bacteria recovered from the middle ear fluid specimens of the HMW1-immunized animals were uniformly downregulated in expression of HMW1, with normal levels of HMW2 expression (data not shown). This downregulation of HMW1 expression was again associated with loss of the ability of the HMW1-immunized chinchilla sera to agglutinate such strains.

DISCUSSION

The components of the protective host immune response to nontypeable *H. influenzae* infection have yet to be fully defined (3, 16, 24, 30). Bacterial outer membrane proteins have been demonstrated experimentally to be potentially important protective antigens (3, 16, 24, 36). Several individual outer membrane proteins have been shown to be targets of bactericidal antibody or targets of protective antibody in animal models of infection. The P2 and P6 proteins of nontypeable *H. influenzae* have both been shown to be targets of human bactericidal antibody (28, 29) and antibody directed against P2 has been demonstrated to enhance pulmonary clearance of nontypeable *H. influenzae* in an animal model of infection (22). However, from the standpoint of possible vaccine development, the P2 protein is poorly immunogenic in infants recovering from acute otitis media (4), and P2 also demonstrates significant strain heterogeneity (27, 30) as well as potential instability (20, 21). The P6 protein is highly conserved and remains an attractive vaccine candidate (17, 29, 30). Recombinant P6 protein that is truncated at the amino terminus and no longer fatty acylated has also been prepared, and it too elicits bactericidal antibody and is protective against infection in a rat bacteremia model (18). A related protein known as PAL (P6) cross-reactive protein or PCP also is reported to be highly conserved and to elicit bactericidal antibody (11). Unfortunately, P6 and other low-molecular-weight proteins offered little if any protection when evaluated in the chinchilla otitis model (19). In addition, P6 appears to be poorly immunogenic in children with recurrent otitis media, a group that would be a prime target for a nontypeable *Haemophilus* vaccine (43). Thus, studies continue to define additional protective antigens.

Attachment of nontypeable *H. influenzae* to the epithelial surface of the upper respiratory tract is a critical first step in colonization of the human host. If one had the ability to interrupt this colonization process, it might also be possible to prevent nontypeable *Haemophilus* disease. For a number of bacteria, adhesin-based subunit vaccines have demonstrated efficacy in protecting against natural infection (7, 26, 32). Vaccines based upon adhesins critical to the nontypeable *H. influenzae* colonization process would also be very attractive candidates for prevention of disease. Having identified the HMW1/HMW2 family of high-molecular-weight proteins as critical adhesion molecules for nontypeable *H. influenzae* in vitro (39), we were particularly interested in defining their protective potential. In the work summarized in this paper, I have now demonstrated that these proteins are capable of modifying the course of disease when evaluated in the chinchilla model of nontypeable *Haemophilus* otitis media.

The protection demonstrated by immunization with the HMW1/HMW2 pr teins was not complete. Although an ideal vaccine should be capable of providing long-lasting and absolute protection against disease, the results with the HMW1/HMW2 proteins should still be considered encouraging. No single bacterial antigen purified from nontypeable *H. influenzae* has yet dem nstrated full protection in the chinchilla otitis model. Furthermore, it should be noted that the mod l that I use is a very stringent test of the protective ability of prototype vaccines. Bacteria are injected directly into the middle ear space and proliferate in what is in essence a small abscess cavity. Bacterial requirements for expression of adhesion molecules that allow attachment to the host epithelium, while critical in the natural in vivo setting, may be unnecessary in such an artificial ecologic niche. This may explain why bacterial variants downregulated in high-molecular-weight protein expression appeared so rapidly in the immunized animals that developed infection. A more physiologic model which required the bacteria to adhere to the nasopharyngeal respiratory epithelium and middle ear space might put a greater premium on expression of adhesion molecules. Such a model of otitis media with adenovirus-*H. influenzae* coinfection has recently been described (40) and is worthy of future studies with the high-molecular-weight adhesion proteins.

Nontypeable *H. influenzae* is known to demonstrate substantial strain heterogeneity (31). In the chinchilla model, I have only demonstrated modification of disease caused by the homologous nontypeable *Haemophilus* strain. What are the chances that immunization with the high-molecular-weight proteins purified from a single strain could protect against infection caused by heterologous nontypeable *H. influenzae*? The HMW1/HMW2-like high-molecular-weight proteins are only expressed by 70 to 75% of nontypeable *H. influenzae* (5), so infection caused by an HMW1/HMW2-nonexpressing strain would likely not be affected by antibodies directed against HMW1/HMW2-like proteins. Even among strains which do express HMW1/HMW2-like proteins, size heterogeneity is apparent in the proteins expressed by unrelated strains (5). Whether this size heterogeneity translates into antigenic heterogeneity which would preclude use of the HMW1/HMW2-like proteins as useful vaccine candidates is unclear at this point. However, I have generated several monoclonal antibodies which recognize epitopes on the high-molecular-weight proteins of most or all nontypeable *H. influenzae* strains which express HMW1/HMW2-like proteins (6; unpublished observations). Furthermore, one of these broadly cross-reactive monoclonal antibodies has been shown to recognize surface-accessible epitopes by immunoelectron microscopy (6). Thus, these data suggest that even if the proteins do demonstrate some heterogeneity, regions of the proteins which do express common surface-accessible epitopes can be identified.

Whether protection against nontypeable *H. influenzae* can be achieved by immunization with a single purified bacterial component is unclear. Although immunization with the purified polysaccharides of bacteria such as *H. influenzae* type b and *Streptococcus pneumoniae* is sufficient to confer protective immunity, it is unclear whether the same success can be achieved with nonencapsulated bacterial organisms. Experience with a bacterium such as *Bordetella pertussis* suggests that administering a vaccine consisting of several distinct surface antigens combined in a multiple-component mixture may provide the highest level of protection against disease (26, 32). A similar strategy may ultimately prove most efficacious in prevention of disease caused by nontypeable *H. influenzae* as well.

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